A HIGH RESOLUTION TYPING SYSTEM FOR PATHOGENIC *BORRELIA*

CLAIM TO DOMESTIC PRIORITY

[0001] This application claims priority to US Provisional application Serial No. 60/393,497 entitled A High Resolution Typing System For Pathogenic *Borrelia* filed July 2, 2002, by Paul S. Keim and Jason Farlow, and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention is generally directed to sub-typing *Borrelia* spirochetes, the causative agent of Lyme Disease, and is more specifically directed to PCR amplification of variable number tandem repeat sequences (VNTR) with primer pairs designed to bind specifically to certain VNTR identified in *Borrelia* isolates. Results of the analysis may be compared to results from known *Borrelia* species to determine the sub-type of the species for epidemiological and diagnostic purposes.

BACKGROUND OF THE INVENTION

[0003] Human Lyme Borreliosis (LB) is the most prevalent arthropod-borne infection in temperate climate zones around the world. LB is caused by members of the *Borreliae* spirochetes (30, 19). In 1996, more than 16,000 cases of Lyme Borreliosis were reported in North America totaling 100,000 cases in a 14 year period (9, 10). *Borreliae* spirochetes are 5 to 25 μm long and 0.2 to 0.5 μm wide (24). These organisms are highly motile, microaerophilic, slow-growing, and fastidious (24). Lyme disease is an inflammatory disorder characterized by the skin lesion erythema migrans and the potential development of neurologic, cardiac, and joint abnormalities (24). The three *Borrelia* species that frequently cause Lyme disease in humans are *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (19, 6). Specific *Borrelia* species can cause distinct clinical manifestations of Lyme disease. *B. burgdorferi* can cause arthritis (2, 28). *B. garinii* is known to cause serious neurological manifestations (2, 28). *B. afzelii* causes a distinctive skin condition known as acrodermatitis chronica atrophicans (ACA)

(27). Each of the three *Borrelia* species causes characteristic erythema migrans (EM)(2, 28).

[0004] The taxonomy of *B. burgdorferi* has undergone extensive revision. At present there are 10 species of *B. burgdorferi* sensu lato characterized and subsequently placed within the *B. burgdorferi* complex. *B. burgdorferi* sensu *stricto* is found primarily in North America and Europe (6, 15, 19, 33). *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae* have been isolated throughout Eurasia (33). *B. japonica*, *B. tanukii*, and *B. turdi* are found primarily in Japan (17, 20). *B. andersonii* and *B. bissettii* are predominantly distributed in North America (22, 31). *Ixodes scapularis*, *Ixodes pacificus*, and *Ixodes ricinus* are the three primary tick reservoirs for *B. burgdorferi* sensu lato (5). The tick reservoir hosts include numerous small mammal species and birds (1, 18, 26).

[0005] Members of *B. burgdorferi* sensu lato are genetically diverse. The bacterium possesses the largest number of extra-chromosomal elements, plasmids, of any known bacterial species: nine circular plasmids and 12 linear plasmids (7, 16). *Borrelia* spp. also has some of the smallest bacterial genomes: ~910 Kb. The combined chromosome/plasmid nucleotide content is approximately 1.5 Mb. Although the *Borrelia* genome mostly evolves in a clonal way (12), *OspC* gene studies suggest lateral transfer does exist (11, 13, 23). The mechanisms of these genetic exchanges could be due to whole plasmid lateral transfer or more likely to gene transfer agent (11). The molecular mechanisms responsible for this genetic exchange are presently unknown. The *Borrelia* genome exhibits significant genetic redundancy and carries 161 to 175 paralogous gene families (7). Such families may serve as foci for inter-plasmid homologous recombination. At least one linear plasmid gene is found within each of 107 gene families creating a significant amount of redundancy and an unusually large number of pseudogenes (7). Approximately 90% of *Borrelia*'s plasmid genes show little similarity to genes of other bacteria (7). It is possible these linear plasmids may be in a phase of rapid evolution and may undergo antigenic variation from immune selection.

[0006] Numerous molecular techniques have recently been used to characterize *Borrelia* species including 16S rRNA gene sequence analysis, SDS PAGE, Western blot

analysis, pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting, randomly amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis, fatty acid profile analysis, and serotyping (4, 8, 15, 33). For a more thorough review of the molecular typing methods used in *Borrelia* characterization see Wang et al, 1999 (33). Although a previous study suggests RAPD analysis is effective for strain discrimination within and among *Borrelia* species (34), its utility in determining robust evolutionary relationships remains questionable due to the method's reduced capacity to provide reproducible data crucial for cladistic character analysis.

[0007] Previously, most *Borrelia* analyses have been performed either phenotypically with monoclonal antibodies, DNA sequencing, or small fragment RFLPs. These analyses involved single genes or limited genomic loci, which do not effectively reflect the characteristics of the whole organism. In addition previous studies either were restricted to one species (29) or used a small number of strains (30). A greater resolution and differentiation of species is necessary for sub-typing *Borrelia* species in order to track sources of infection and ultimately to prevent the spread of disease.

[0008] Simple sequence repeats (SSRs) or variable number tandem repeats (VNTRs) have been shown to provide a high level of discriminatory power (21). This stems from the significant mutability of repeat copy number. Multiple-locus VNTR analysis (MLVA) has previously shown great discriminatory capacity and accurate estimation of genetic-relationships within bacterial pathogens such as *Francisella tularensis* and *Bacillus anthracis* (14, 21).

[0009] Methods and means for determining the genetic differences between *Borrelia* species with speed, accuracy and with great discriminatory capacity have been sought.

SUMMARY OF THE INVENTION

[0010] The present invention discloses methods and means for detecting and sub-typing *Borrelia* species by multi-locus analysis of VNTR identified within the genome of *Borrelia burgdorferi*.

[0011] In an important aspect of the present invention, isolated nucleic acids are presented comprising at least 12, 15, 18 or total consecutive nucleotides of a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3; SEQ ID NO: 4; SEQ ID NO: 5; SEQ ID NO: 6; SEQ ID NO: 7; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10. SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16; SEQ ID NO: 17; SEQ ID NO: 18, SEQ ID NO: 19; and SEQ ID NO: 20 and sequences complementary thereto.

[0012] In certain preferred embodiments of the invention, these nucleic acids are immobilized on a solid surface and are useful, for example, in the detection of a Borrelia species in an assay employing probes, including, but not limited to, a nano-detection device.

[0013] In another important aspect of the invention, primer pairs comprising a forward and a reverse primer, are presented for amplification of VNTR located in DNA from a *Borrelia* species. Primer pairs suitable for PCR amplification of VNTR, by MLVA or by multiplex , for example, may be selected from the group consisting of SEQ ID NO 1 and 2, SEQ ID NO: 3 and 4, SEQ ID NO: 5 and 6, SEQ ID NO: 7 and 8 SEQ ID NO: 9 and 10, SEQ ID NO: 11 and 12, SEQ ID NO: 13 and 14, SEQ ID NO: 15 and 16, SEQ ID NO: 17 and 18, and SEQ ID NO: 19 and 20. Certain preferred primer pairs have, in addition, an observable group whereby amplified product may be detected. Such groups may be, for example, a fluorescent group or a radioactive group.

[0014] In yet another important aspect of the invention, a method for detecting a *Borrelia* species is presented. The method comprises the steps of:

i. obtaining a DNA sample from said species,
ii. amplifying a VNTR marker loci in said DNA with one or more primer pairs; and
iii. detecting an amplification product that contains the VNTR sequence.

[0015] In another important aspect of the invention, MLVA methods are presented for observing polymorphisms at VNTR loci in DNA from more than one *Borrelia* species to resolve unique genotypes between the species and to allow sub-typing of the species into distinct groups. These MLVA methods provide a convenient and rapid method for strain discrimination in *Borrelia*. MLVA may be applied for strain discrimination among globally diverse *Borrelia* isolates including *B. burgdorferi, B. afzelii,* and *B. garinii.*

[0016] In yet another important aspect of the invention, kits are provided for detecting and sub-typing Borrelia species. The kits comprise one or more primer pairs suitable for amplifying VNTR in DNA in a sample of said species and may comprise, in addition, nucleic acids, enzymes, tag polymerase, for example, and buffers suitable for causing amplification by PCR, by MLVA or by multiplex, for example. In certain preferred embodiments of the kit the primers comprise a label whereby amplified VNTR may be detected. In other preferred embodiments of the kit, labeled nucleic acids are provided. Observable labels are preferably fluorescent molecules or radionucleotides.

[0017] A method of sub-typing a *Borrelia* strain is provided comprising the steps of:

i. obtaining DNA from said strain;
ii. amplifying said DNA with one or more primer pairs selected from the goup consisting of SEQ ID NOS: 1-20;
iii. detecting said amplified product;
iv. determining the diversity number of said amplified product; and
v. comparing said diversity number with the diversity number for a known strain of *Borrelia.*

BRIEF DESCRIPTION OF THE FIGURES

[0018] Figure 1 illustrates genetic relationships among *Borrelia* isolates. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis based upon allelic differences from ten VNTR markers across 41 *B. burgdorferi, B. afzelii,* and

B. garinii isolates was used to construct this dendogram. Letters to the right of each branch correspond to the individual sample identification (Table 2) followed by *Borrelia* species designation. The horizontal axis indicates estimated VNTR allelic differences (Allelic differences are a measure of genetic evolutionary distance). Roman numerals indicate arbitrary groupings of species.

[0019] Figure 2 illustrates the correlation between repeat copy number and diversity measures. The B31 *B. burgdorferi* strain repeat copy number (Table 1) was compared diversity (Pearson coefficient R = 0.62) and total observed allele number (Pearson coefficient R = 0.94) at each marker locus. Crosses (+) indicate the marker's total observed allele number versus repeat copy number at an individual marker locus. Diamonds (♦) indicate the marker's calculated diversity value versus the repeat copy number of an individual marker. Analysis was performed using only data from the eight *Borrelia* markers with non-complex repeat motifs.

DETAILS OF THE INVENTION

[0020] The present invention discloses the successful application of MLVA for strain discrimination among globally diverse *Borrelia* isolates including *B. burgdorferi, B. afzelii,* and *B. garinii.* Ten VNTR loci have been identified from genomic and plasmid sequences of *Borrelia* strains (Table 3, Marker locus number BR-V1 to BR-V10) Polymorphisms at these loci were may be used to resolve genotypes into distinct groups. Figure 1 is a dendogram illustrating the resolution of 30 unique genotypes into five to seven distinct groups. This sub-typing scheme is useful for the epidemiological study of *Borrelia* and may be applied to the local detection of the pathological causative agent of Lyme Disease.

[0021] The following definitions are used herein:

[0022] "Polymerase chain reaction" or "PCR" a technique in which cycles of denaturation, annealing with primer, and extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence by approximately 106 times or

more. The polymerase chain reaction process for amplifying nucleic acid is disclosed in US Pat. Nos. 4,683,195 and 4,683,202, which are incorporated herein by reference.

[0023] "Primer" a single-stranded oligonucleotide or DNA fragment which hybridizes with a DNA strand of a locus in such a manner that the 3' terminus of the primer may act as a site of polymerization using a DNA polymerase enzyme.

[0024] "Primer pair" two primers including, primer 1 that hybridizes to a single strand at one end of the DNA sequence to be amplified and primer 2 that hybridizes with the other end on the complementary strand of the DNA sequence to be amplified.

[0025] "Primer site": the area of the target DNA to which a primer hybridizes.

[0026] "Multiplexing" is a capability to perform simultaneous, multiple determinations in a single assay process and a process to implement such a capability in a process is a "multiplexed assay." Systems containing several loci are called *multiplex* systems described, for example, in US Patent No. 6,479,235 to Schumm, et al., US Patent No. 6,270,973 to Lewis, et al. and 6,449,562 to Chandler, et al.

[0027] Isolated nucleic acid" is a nucleic acid which may or may not be identical to that of a naturally occurring nucleic acid. When "isolated nucleic acid" is used to describe a primer, the nucleic acid is not identical to the structure of a naturally occurring nucleic acid spanning at least the length of a gene. The primers herein have been designed to bind to sequences flanking VNTR loci in *Borrelia* species. It is to be understood that primer sequences containing insertions or deletions in these disclosed sequences that do not impair the binding of the primers to these flanking sequences are also intended to be incorporated into the present invention.

[0028] The present invention provides primer pairs for PCR amplification of VNTR in DNA of *Borrelia.* The primer pairs comprise a forward primer and a reverse primer. Table 1 illustrates the Borrelia Primer Sequences of the present invention.

Table 1. *Borrelia* Primer Sequence

Marker Name	Forward sequence	Reverse sequence
BR-V1	GTTCAAGATATGGTTAAGGGCAATTTAGATAAAGATC	GAAGACTTACATGCCAGTTCATCAAGAGTC
BR-V2	GTATAATGAAGTTAGTGGGCGTTACTCTTGGGTAC	GAAACCATAAAACCATCTAAAGATACAAATCATTC
BR-V3	GTTTGTCGTTGCCAAAACTGCTTTCATAATTC	GGGATTAAATATGAAAATATATTTAGTTTGTGTGCATTATATCTGC
BR-V4	GTTTCTGCGACTAGGTATGGAACAACTAATAGCTC	GCAGTGGGCACAACTACTACTGCAATAATAACTAC
BR-V5	GCAATCCAAAATATTCAAGATCGTATAAAAATGTC	GATGATAAAATTTTCAAATGTATATCTTTTTTTAAGAAAGGC
BR-V6	GGATCGATCGTACTGTGCAGCCACAAACGTGCTGCGC	GTAGCGTACGTAGCTGCGCGTAGTATTTTTATCGTAGCGCGAGC
BR-V7	GCTTCAAAATGCTGCTTCAATTGCTGGAC	GCAAAAACACAAGCTTGCCGGTGAAAC
BR-V8	GATCTAATTCATTAAAAAATTTTGTGAAAGGGGCTTC	GATAAATAACTTGCAATATTTCCGCTTAAGGTAGTTTTC
BR-V9	GTCATCTTTAGTGTCTAATTTTAGAATTTTATTAACTTTTTCTTTGC	GTCATGCTTATATCAATGCCCTATGCCTCAAC
BR-V10	GCTTTTAACGCTAAATTATAAAGAAAAATTATTTCATTTCGGC	GTCAAAATTATGCTTCCAAAAGCATTACAATTAAAAAAATC

These primer sequences have herein been assigned SEQ ID NO: as follows:

SEQ ID NO	Marker Name	
SEQ ID NO: 1	BR-V1	Forward primer
SEQ ID NO: 2	BR-V1	Reverse primer
SEQ ID NO: 3	BR-V2	Forward primer
SEQ ID NO: 4	BR-V2	Reverse primer
SEQ ID NO: 5	BR-V3	Forward primer
SEQ ID NO: 6	BR-V3	Reverse primer
SEQ ID NO: 7	BR-V4	Forward primer
SEQ ID NO: 8	BR-V4	Reverse primer
SEQ ID NO: 9	BR-V5	Forward primer
SEQ ID NO: 10	BR-V5	Reverse primer

SEQ ID NO: 11	BR-V6	Forward primer
SEQ ID NO: 12	BR-V6	Reverse primer
SEQ ID NO: 13	BR-V7	Forward primer
SEQ ID NO: 14	BR-V7	Reverse primer
SEQ ID NO: 15	BR-V8	Forward primer
SEQ ID NO: 16	BR-V8	Reverse primer
SEQ ID NO: 17	BR-V9	Forward primer
SEQ ID NO: 18	BR-V9	Reverse primer
SEQ ID NO: 19	BR-V10	Forward primer
SEQ ID NO: 20	BR-V10	Reverse primer

[0029] The polynucleotides of the present invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art. The availability of nucleotide sequence information enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire long double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vecto

[0030] Methods for using these primer pairs to amplify VNTR loci in *Borrelia* are disclosed herein. Generally MLVA analyses or multiplex systems known to the art may

be employed to detect and sub-type *Borrelia.* PCR instruments used in these amplification methods are commercially available.

[0031] Kits are herein provided for use with commercially available PCR instruments to detect and sub-type strains of *Borrelia.* The kits contain one or more primer pairs disclosed hereinabove having SEQ ID NOS 1-20 for amplifying the VNTR in DNA isolated from a *Borrelia* sample. If the sample is to be multiplexed, the kits may contain a suitable "cocktail" of primer pairs.

[0032] The kits may also contain nucleic acids needed in the amplification process. The nucleic acids may be tagged by a suitable marker, a fluorescent probe or a radioactive molecule. Any tag for marking the nucleic acid after amplification and size separation as by electrophoresis or other separation means is suitable. In certain preferred embodiments of the invention, the primer pairs themselves comprise a suitable marker.

[0033] The kits may also comprise enzymes, taq polymerase, for example and salts and buffers suitable for causing amplification of DNA by PCR. This kits may also comprise suitable containers and bottles for housing these reagents and or convenient use.

[0034] Kits for sub-typing strains of *Borrelia* comprise, in addition, DNA isolated from known *Borrelia* strains. This isolated DNA containing VNTR loci may be used as standards in the sub-typing of the species.

EXPERIMENTAL DETAILS

[0035] **Genomic** analysis. The *B. burgdorferi* sensu stricto B31 strain genomic sequence was downloaded from the NCBI web page (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?gi=132&db=Genome) and used to identify potential VNTR loci. Sequences were screened from the 946 Kb genome, the 12 linear plasmids, and the nine circular plasmid of *B. burgdorferi.* Each sequence was screened for the presence of tandem repeats using the DNAstar software program Genequest (Lasergene, Inc. - Madison, WI). This program locates and displays tandem and non-tandemly repeated

arrays. Confirmation of the repeated sequence structure was performed using dot plot similarity analysis in the software program Megalign (Lasergene, Inc. –Madison WI).

[0036] **PCR amplification of VNTR loci.** MLVA primers were developed around 46 potential VNTR loci using the DNA Star program PrimerSelect. A total of 10 primer sets amplified polymorphic VNTR loci (Table 1) while 36 loci proved monomorphic. Reagents used in the PCR reactions were obtained from Life Technologies. Primers were designed with annealing temperatures from 65°C to 61°C. Individual primer pair annealing temperatures were designed within 2°C of each other.

[0037] Bacterial **thermolysates.** *Borrelia* strains were grown in BSK medium (Sigma) until they reached 10^7 bacteria/ml. One ml was harvested by centrifugation, washed in PBS and re-suspended in 100µl of water before heating at 100°C for 20 minutes.

[0038] **Automated genotyping.** Fluorescently labeled amplicons were sized by polyacrylamide gel electrophoresis (PAGE) in an ABI377 DNA Sequencer. Analysis was accomplished using the Genescan and Genotyper software (14). The PCR product was diluted three-fold and mixed 1:1 with equal parts of a 5:1 formamide:dextran blue dye and size standard prior to electrophoresis. The Bioventures Rox 1000 size standard was used with filter set D.

[0039] **Statistical analysis.** Pairwise genetic differences among isolates were estimated using a simple matching coefficient. The clustering method used to evaluate genetic relationships was Un-weighted Pair Group Method with Arithmetic mean (UPGMA) in the software PAUP4a (D. Swofford, Sinauer Associates, Inc., Publishers, Sunderland MA) The diversity (D) for each marker was calculated as [1 - Σ(allele frequencies)^2] (34).

[0040] **VNTR marker identification and diversity.** Analysis of the genomic sequence of *B. burgdorferi* type strain B31 revealed 225 genomic sequence motifs that potentially represent VNTR loci. An additional 167 potential VNTR loci were identified among the plasmid sequences of *B. burgdorferi* (type strain B31 46 repeated sequence

motifs were chosen from these for MLVA analysis. MLVA revealed that 36 were monomorphic and only ten proved to be polymorphic loci (Table 3) among 41 globally diverse *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains (Table 2). However, all loci did not support PCR amplification. A total of 19 isolates failed to yield PCR products across markers BR-V4, 6, 8, and 10 (Table 4). Sixteen of these 19 failures occurred within plasmid-based loci (Table 4).

Table 2. *Borrelia* Strain Information

Strain ID	*Borrelia* species	Country	Source	Provided by
ESP1	*burgdorferi*	Spain	*I. Ricinus*	R.C. Johnson
SON328	*burgdorferi*	USA California	*I. Pacificus*	M. Janda
IP2	*burgdorferi*	France (Tours)	Human CSF	G. Baranton
SON2110	*burgdorferi*	USA California	*I. pacificus*	M. Janda
HB19	*burgdorferi*	USA Connecticut	Human blood	A. Barbour
IP1	*burgdorferi*	France (Poitiers)	Human CSF	G. Baranton
B31	*burgdorferi*	USA New York	*I. scapularis*	ATCC35210
ZS7	*burgdorferi*	Germany	*I. ricinus*	L. Gern
20006	*burgdorferi*	France	*I. ricinus*	J.F. Anderson
VEERY	*burgdorferi*	USA Connecticut	Veery bird	R.T. Marconi
MEN115	*burgdorferi*	USA California	*I. pacificus*	M. Janda
CA19	*burgdorferi*	USA California	*I. pacificus*	T. Schwan
19535	*burgdorferi*	USA New York	*Peromyscuc leucopus*	J.F. Anderson
MIL	*burgdorferi*	Slovakia	*I. ricinus*	A. Livesley
Cat Flea	*burgdorferi*	USA Texas	*Ctenocephalides felis*	D. Ralph
21305	*burgdorferi*	USA Connecticut	*Peromyscuc leucopus*	J.F. Anderson
NY186	*burgdorferi*	USA New York	Human skin	R.T. Marconi
DK7	*burgdorferi*	Denmark	Human skin	M. Theisen
297	*burgdorferi*	USA Connecticut	Human CSF	R.C. Johnson
26816	*burgdorferi*	USA Rhode Island	*Microtus pennsylvanicus*	J.F. Anderson
SON188	*burgdorferi*	USA California	*I. pacificus*	M. Janda
IP3	*burgdorferi*	France (Pau)	Human CSF	G. Baranton
Z136	*burgdorferi*	Germany	*I. ricinus*.	A. Vogt

35B808	*burgdorferi*	Germany	*I. ricinus*	A. Schönberg
NE56	*burgdorferi*	Switzerland	*I. ricinus*	L. Gern
27985	*burgdorferi*	USA Shelter Island	*I. scapularis*	J.F. Anderson
L5	*burgdorferi*	Austria	Human skin	G. Stanek
DK3	*afzelii*	Denmark	Human skin	R.C. Johnson
BR53	*afzelii*	Czeck Republic	*Aedes vexans*	Z. Hubalek
ECM1	*afzelii*	Sweden	Human skin (EM)	S. Bergstrom R.T. Marconi
J1	*afzelii*	Japan	*I. persulcatus*	
B023	*afzelii*	Germany	Human skin (ECM)	A. Vogt
VS461	*afzelii*	Switzerland	*I. ricinus*	O. Peter
DK8	*afzelii*	Denmark	Human skin	R. C. Johnson
PBI	*garinii*	Germany	Human CSF	C. Kodner
VSDA	*garinii*	Switzerland	Human CSF	O. Peter
N34	*garinii*	Germany	*I. ricinus*	J. Ackerman
20047	*garinii*	France	*I. ricinus*	J.F. Anderson
HFOX	*garinii*	Japan	Fox (heart)	E. Isogai
PBR	*garinii*	Germany	Human CSF	B. Wilske
FAR03	*garinii*	Sweden	Seabird	S. Bergström

[0041] The ultimate utility of VNTR loci lies in their diversity. The present invention discloses the use of marker diversity using both allele number and frequency to sub-type *Borrelia* species. The allele number observed ranged from two (BR-V7) to nine alleles (BR-V8) (Table 3). The larger the repeat array in the B31 strain, the greater the VNTR diversity (R=0.62) and number of alleles (R=0.94) among globally diverse strains (Figure 2). For example, marker BR-V8 has a repeat copy number of 8.3, in the B31 type strain, and exhibits 9 alleles (Table 3). In contrast, marker BR-V9 with a copy number of only three exhibits only three alleles in our study (Table 3). Repeat motifs were obgserved ranging from two base pairs for BR-V3 to 21 base pairs for BR-V8 (Table 3). Minimum array size observed across all alleles ranged from one (BR-V10) to 29 (BR-V3) (Table 3). Diversity index values (D) ranged from 0.1 to 0.89 with an overall average

diversity index value of 0.51 (Table 3). VNTR markers that exhibit high diversity values such as BR-V8 (D=0.89) possess great discriminatory capacity for identifying genetically similar strains. Less diverse markers such as BR-V9 (D = 0.10) (Table 3), may be applied with greater utility in species identification and the analysis of evolutionary relationships. This demonstrated ability to predict VNTR diversity based upon array size allows the guided selection of marker loci.

Table 3. VNTR Marker Attributes

Marker Locus	Repeat Motif	Genome/Plasmid Coordinate[a]	Repeat Size (nucleotides)	*Borrelia* s. Array Size	Smallest Array Size	Largest Array Size	Number of Alleles	Diversity[b] D
BR-V1	Complex array	CH-844,650	CX[c]				6	0.74
BR-V2	TAAAT	CH-590,955	5	5	8	11	4	0.67
BR-V3	TA	LP17-10,530	2	5	22	29	4	0.14
BR-V4	Complex array	LP28-2-28,142	CX[d]				8	0.55
BR-V5	AAG	CH-456,964	3	4	2	4	3	0.63
BR-V6	TGA	CH-720,032	3	4	1	3	3	0.51
BR-V7	TGC	CH-690,090	3	4	13	14	2	0.1
BR-V8	*	LP17-13,155	21	8.3	6	14	9	0.89
BR-V9	TTC AATATTAA	LP28-3-4,235	3	4	3	4	3	0.1
BR-V10	ATA	LP54-20,145	11	5.5	1	9	7	0.75
						Average =	4.9	0.51

[a] = CH indicates chromosome locus, LP indicates linear plasmid locus

[b] D = 1 - sum(allele frequency)2

[c] = CX indicates the complex nature of the repeat motif and consequently makes accurate array size calculation difficult. The B31 sequence at this locus consists of four tandem repeats. For example, a 32 base pair motif repeated 2.2 times is listed here in the form (32 x 2.2). Other arrays that contribute to the complexity observed at this locus include the following: (32 x 3.2) + (32 x 2.0) + (41 x 2.0)

[d] = (86 x 2.2) + (32 x 4.0) + (32 x 2.6)

* indicates the 21 bp repeat TAATTAATATGTGATATAAAA

[0042] **Genetic Relationships among isolates.** Ten VNTR marker loci were used to calculate genetic distances among the *Borrelia* strains. UPGMA analysis then revealed 30 distinct genotypes among the 41 *Borrelia* isolates with five unique subdivisions evident within these affiliations (Figure 1). No fixed allelic differences were present between these clusters (Table 4), therefore cluster formation is due to overall allelic frequency. Cluster I, II, III, and IV include only *B. burgdorferi* sensu stricto isolates (Figure 1). All *B. burgdorferi* strains revealed unique marker allele-size combinations, with the exception of *B. burgdorferi* strains L5, IP1, IP2, IP3, Cat flea and B31 which were identical at all marker loci (Figure 1). Isolates B31 and Cat flea were isolated in North America, while strains IP1, IP2, and IP3 are human CSF isolates from France (Table 2). A total of 19 of the 27 *B. burgdorferi* sensu stricto strains grouped within cluster IV (Figure 1). MLVA revealed substantial discrimination between *B. afzelii* and *B. garinii* evident in cluster V (Figure 1). This cluster included seven *B. afzelii* strains and seven *B. garinii* strains (Figure 1). All seven *B. afzelii* strains assembled within the single sub-group of cluster V-1 (Figure 1). *B. afzelii* isolates B023 and BR53 showed 100% marker identity as did isolates J1, ECM1, DK3, DK8, and VS461 (Figure 1). Six unique genotypes are evident among the *B. garinii* isolates with strains Far03 and VSDA showing 100% marker identity (Figure 1). Although the Japanese *B. garinii* strain (HFOX) loosely clustered within the *B. afzelii* subgroup (Figure 1), this strain exhibits only a single *B. afzelii*-specific chromosomal allelic state (Table 4). The HFOX isolate also exhibits a *B. burgdorferi*-specific plasmidic allele and a unique allele specific to this isolate alone (Table 4). The loose affiliation of HFOX with *B. afzelii* (cluster V-1, Figure 1) does not appear robust. This affiliation is not contradictory to the identity of HFOX in this un-rooted tree, as HFOX is more closely related to the *B. garinii* isolates than to the *B. afzelii* isolates. Overall, the phylogenetic relationships observed in this study are in general agreement with previous 16S rRNA sequence analysis (31) with the *Borrelia* MLVA system developed here providing greater capability for individual strain discrimination.

Table 4. *Borrelia* Alleles

Marker Loci

Strain ID	BR-V1	BR-V2	BR-V3	BR-V4	BR-V5	BR-V6	BR-V7	BR-V8	BR-V9	BR-V10
SON188	706	173	144	522	116	89	206	300	201	476
NY186	800	178	144	522	116	89	206	321	204	476
MIL	750	173	144	697	116	89	206	404	204	465
MEN115	800	178	144	697	116	89	206	384	204	465
LS	800	178	144	697	116	89	206	321	204	509
IP1	800	178	144	697	116	89	206	321	204	509
IP2	800	178	144	697	116	89	206	321	204	509
ESP1	750	173	150	697	119	89	206	342	204	454
IP3	800	178	144	697	116	89	206	321	204	509
DK7	800	178	144	697	119	89	206	279	204	465
Cat Flea	800	178	144	697	116	89	206	321	204	509
19535	800	178	144	697	116	*	206	321	204	465
20006	750	178	144	697	119	89	206	363	204	454
B31	800	178	144	697	116	89	206	321	204	509
SON2110	750	183	144	638	116	89	204	300	204	476
SON328	750	173	144	638	119	86	206	363	204	542
CA19	750	173	144	522	116	86	204	285	204	454
297	750	173	144	802	116	86	206	454	204	465
21305	750	178	144	835	116	*	206	404	204	*
VEERY	750	178	144	*	119	89	206	321	204	520
27985	800	178	144	697	116	89	206	300	204	465
ZS7	800	178	144	642	119	89	206	300	204	454
HB19	750	178	144	608	116	89	206	300	207	465
35B808	800	173	144	697	119	89	206	300	204	465
Z136	706	173	144	697	116	89	206	384	204	608
26816	800	178	144	697	116	89	206	342	204	509
NE56	750	183	154	697	119	89	206	363	204	454

B023	750	168	144	697	113	68	206	*	204	465
J1	706	168	144	697	113	68	206	*	204	465
ECM1	706	168	144	697	113	*	206	*	204	*
DK3	706	168	144	697	113	68	206	300	204	465
DK8	706	168	144	697	113	68	206	*	204	465
BR53	750	168	144	697	113	68	206	*	204	*
VS461	706	168	144	697	113	68	206	*	204	465
20047	655	168	144	697	113	89	206	321	204	509
N34	655	168	142	697	113	89	206	342	204	465
FAR03	692	168	144	731	113	89	206	*	204	465
VSDA	692	168	144	*	113	89	206	*	204	465
PBI	655	168	144	638	113	89	206	363	204	*
PBR	692	168	144	697	113	89	206	*	204	465
HFOX	467	168	144	802	113	68	206	*	204	465

*Missing data due to lack of PCR amplification

Scores indicate allele sizes in base pairs.

[0043] The diversity within the three species is dramatically different and suggests phylogenetic relationships and evolutionary history. For example, we observed that four out of the five clusters contain only *B. burgdorferi* sensu stricto members. These four groups have great diversity, especially when contrasted with the *B. afzelii* (group V-1, Figure 1) and *B. garinii* (V-2, Figure 1) cluster. The cohesiveness of these two latter species into one group argues for a more recent common evolutionary derivation, perhaps from a *B. burgdorferi* sensu stricto ancestor. Certainly their lack of diversity is due to either a recent origin or a common and pronounced genetic bottleneck.

[0044] A more subtle diversity trend is observed within *B. burgdorferi* sensu stricto when North America and European strains are compared (Fig. 2). We observed greater genetic diversity among the 15 North American samples (mean genetic distance = 0.46) versus that among 12 European samples (mean genetic distance = 0.41). Perhaps due to a relatively small sample size this trend is not statistically significant (t = 0.009) but it is consistent with previous evolutionary models postulating a founder effect as North American *B. burgdorferi* sensu stricto moved to the Old World (15, 23). However, diversity within *B. burgdorferi* sensu stricto could likewise be affected by lateral transfer of genetic material from other species. In previous studies, four diverse isolates (NE56, 20006, Z136, ESP1) were shown to have obtained the *ospC* gene from other species (23). Hence genetic mixing via lateral transfer may provide an additional mechanism for evolutionary change.

[0045] The characterization of molecular diversity with MLVA analysis to the strain-typing of *B. burgdorferi, afzelii*, and *garinii*, suggests this method can be harnessed for the rapid discrimination and identification of remaining major *Borrelia* species and allow for further phylogenetic and epidemiological analysis of this genetically diverse organism.

EXAMPLES

EXAMPLE 1

[0046] This example illustrates PCR amplification of the ten variable loci from 41 *Borrelia* isolates.

[0047] 2mM $MgCl_2$, 1X PCR buffer, 0.1mM dNTPs, 1μM R110, R6G, or Tamra phosphoramide fluorescent labeled dUTPs (Perkin Elmer Biosystems), 0.5 units of Taq polymerase, 1.0 μL template DNA, 0.5 μM forward primer, 0.5 μM reverse primer were combined in filtered sterile water to a volume of 12.5 μL. The reaction mixtures were incubated at 94°C for 5 minutes in the PCR instrument (a commercially available thermocycler) and then cycled at 94°C for 30 seconds, 61°C or 56°C for 30 seconds,

72°C for 30 seconds and 94°C for 30 seconds for 35 cycles, with a final incubation of 72°C for 5 minutes.

EXAMPLE 2

[0048] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V1 with primer pairs SEQ ID NO: 1 and SEQ ID NO: 2.

GGCTTAGTTTTGAGTTTGAGGAGGAGTAGGTTTGTCGAAAAATATTGATG
ATATAAAAATGAAGATGGCAAAAAAGTTAAGATTATTAAGTTGAAAAAGAA
GGTAGTAAAAATTGTAACATATAATGATTTAAGCGTTAAAAATGATTCAAAT
AGCTTTGTTGATTTGCATAATAACAGCAATAAGGCTGAATATTCGCAAAGTA
GAGACAATAGAACTGGCGGGTATTCACAAAATAGGGACAATAGAGCTGGTG
GATATTCACAAAATAGGGACAATAGAGCTGGTGGATATTCCCAAAACAGAGA
CAACAGAACTGGTGGGTATTCACAAAACAGAGACAACAGAACTGGTGGGTA
TTCACAAAACAGAGACAACAGAACTGGTGGGTATTCACAAAATAGGGATAAT
AGAGGTGGATATTCACAAGGCAGAGACAACAGAACTGGTGGATATTCACAA
AGCAGGGACAATAGAACTGGTGGATATTCACAAAACAGGGACAATAGAACT
GGTGGATATTCACAAAACAGAGACAATAGAACTGGTGGATATTCACAAAACA
GAGATAACAGAACTGGTGGATATTCACAAAACAGAGACAGCTTATCCTTTCA
ATATCAAGGTTCAGTAAAGAAAACATATGTTGCCAAAAATAATTCTCAAAAT
AAATATACTACTACTTCTATGTCTTTTAGAAGACTTATAAAAACTAAAGTTCC
CGCTATT**GTTAGCAGCACACCTGCAGCGGATTC**

EXAMPLE 3

[0049] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V2 with primer pairs SEQ ID NO: 3 and SEQ ID NO: 4.

GTATAATGAAGTTAGTGGGCGTTACTCTTGGGTAAAAAGAAAGTAAATTT
AATTTAAAATTAGTTTTAAATTAAATTAAATTAAATTAAATGAGGA**GAATGA**
TTTGTATCTTTAGATGGTTTTATGGTTTC

EXAMPLE 4

[0050] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V3 with primer pairs SEQ ID NO: 5 and SEQ ID NO: 6.

GTTTGTCGTTGCCAAAACTGCTTTCATAATTCACTCACCTACTATATATAT
ATTTTAACATAAATCAAAGCCAAATATCGGAACATTTCCTTCAAAATCTCATA
AA**GCAGATATAATGCACACAAACTAAATATATTTTCATATTTAATCC**

EXAMPLE 5

[0051] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V4 with primer pairs SEQ ID NO: 7 and SEQ ID NO: 8.

GTTTCTGCGACTAGGGTATGGAACAACTAATAGCTCAAGATTTATCAAAA
AATATTATCACAATGAACTTACATATAGAGATTTAGAAAATTTAGAAAAGCA
ATTTGGCATAAAGTTTGACAATCTTGTTACTAAGATTGATACTGTTAAAAGTG
AACTTACTACTAAGATTGATAATGTAGAAAAGAATTTACAAAAGGATATATC
CAACTTAGACGTTAAGATTGATACTGTTAAAAGTGAACTTACTACTAAGATTG
ATAACGTAGAAAAGAATTTACAAAAGGATATATCCAACTTAGACGTTAAGAT
TGATACTGTTAAAAGTGAACTACTACTAAGATTGATAACGTAGAAAAGAAT
TTAGATACTAAGATTGATAACGTAGAAAAGAATTTAGATACTAAGATTGATA
ACGTAGAAAAGAATTTAGATACTAAGATTGATAACGTAGAAAAGAATTTGCA
AAAAGATATGTTTAGTTTGGAACAAAGGCTAGAAATAAAGCTGGAAGCCAAT
AACAAACTTCTTTTGGAAAAGCTGGAAGCCAATAACAAACTTCTTTTGGAAA
AGCTGGAAGCCAATAGCAAAGTTCTTTTGGAAAAGCTAGAAGCCAATAACAA
AGTTTCTTCAGAAAAGCTTAAAGTCAGCAACAGAAGTAGTTATTATTGCAG
TAGTAGTTGTGCCCACTGC

EXAMPLE 6

[0052] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V5 with primer pairs SEQ ID NO: 9 and SEQ ID NO: 10.

GCAATCCAAAATATTCAAGATCGTATAAAAATGTATATCAAAAAAGAAGA
AGAAGAGCCCACAAATTTTAAAAACCCCTTTCTTAAAAAAAGATATACA
TTTGAAAATTTTATC

EXAMPLE 7

[0053] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V6 with primer pairs SEQ ID NO: 11 and SEQ ID NO: 12.

GTTCAAGATATGGTTAAGGGCAATTTAGATAAAGATTATGCTCTTGATGAT
GATGAAAATACTCTTGATGAACTTGGCATGTTAAGTCTTC

EXAMPLE 8

[0054] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V7 with primer pairs SEQ ID NO: 13 and SEQ ID NO: 14.

GCTTCAAAATGCTGCTTCAATTGCTGGACTTTTATTAACAACAGAATGTGC
AATCACAGATATTAAAGAAGAGAAAAATACTTCTGGTGGTGGTGGTTATCCT
ATGGACCCAGGAATGGGAATGATGTAAATTAAA**GTTTCACCGGCAAGCTTG**
TGTTTTTGC

EXAMPLE 9

[0055] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V8 with primer pairs SEQ ID NO 15 and SEQ ID NO 16.

GATCTAATTCATTAAAAATTTTGTGAAAGGGGCTTCATAGGTAGAGATT
AAAGTAATTAATATGTGATATAAAATAATTAATATGTGATATAAAATAATTA
ATATGTGATATAAAATAATTAATATGTGATATAAAATAATTAATATGTGATAT
AAAATAATTAATATGTGATATAAAATAATTAATATGTGATATAAAATAATTA
ATATGTGATATAAAATAATTAAAAGGAAGTTTGTATGAAAAAATAGCATTT
CATATTCAAAAGGTGGTGTTGG**GAAAACTACCTTAAGCGGAAATATTGCA**
AGTTATTTATC

EXAMPLE 10

[0056] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V9 with primer pairs SEQ ID NO 17 and SEQ ID NO 18.

GTCATCTTTAGTGTCTAATTTTAGAATTTTATTAACTTTTTCTTTGCTAAA
TTTAAAATGCTCTAAGTAAAGCAAATTAGAGAAATTTAAAGGATCATTTTTA
GCTATTAACAAGGAAGTGTTTTTTACTAAAGTTAAGTATATCGGATTAGCTAA
AATTTCTTCTTCTTCGG**GTTGAGGCATAGGGCATTGATATAAGCATGAC**

EXAMPLE 11

[0057] This example illustrates the amplicon produced during the amplification of VNTR locus BR-V10 with primer pairs SEQ ID NO 19 and SEQ ID NO 20.

GCTTTTAACGCTAAATTATAAAGAAAAATTATTTCATTTCGGCAGGTTTAA
ATATTTATAATTTTATCTAAATTTTTAGTTATCAATTTAAGTTTATTGTAATTA
ATAATTGTTTAAAAGTTTTTGTCTTTTGTGCTGATTTTGCTAAAAATTCTTTTT
TGCCTTGAATTTAGGCTAAATCAATATTAAATCAATATTAAATAAATATTAAA
TAAATATTAAATAAATATTAAATAAATATTAAATAAATATTTAAAACAATTA
AATTTTTATATTAAAAAATGCAAATTTTTGTAAAAAATATAAAATTAATATTC
AATCTTTTAAAGATTTTGAAAAATTTTTTTTAAAGTTTATTTTTTTGGAAAATA
TTATTGATATGATTTATAATTTAATTTTTATTATTTTACCACTAAGGAGTCTAT
TATGAAAAACAGATTTTTTCTATATTTTTGTTCTTCAAACTTTGATTTTTTTAA
T TGTAATGCTTTTGGAAGCATAATTTTGAC

[0058] While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

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